

**IN THE SPECIFICATION:**

Page 13, before line 24, please add the following paragraph:

-- DNA encoding antibody scFv L-19 has been deposited on September 25, 2008, in ATCC (Manassas, VA), and has accession no. PTA-9529.

Page 15, please replace the paragraph beginning at line 14, with the following:

--2D gels and western blotting (a) Silver-staining of the 2D-PAGE of a lysate of human melanoma COLO-38 cells, to which recombinant ED-B-containing 7B89 had been added. The two 7B89 spots (circle) are due to partial proteolysis of the His-tag used for protein purification. (b) Immunoblot of a gel, identical to the one of Fig. 2a, using the anti-ED-B E1 (~~Table 1~~) and the M2 anti-FLAG antibodies as detecting reagent. Only the 7B89 spots are detected, confirming the specificity of the recombinant antibody isolated from a gel spot.--.

Page 21, please replace the paragraph beginning at line 8, with the following:

--In order to test whether the antibodies isolated against a gel spot had a good affinity towards the native antigen, real-time interaction analysis was performed using surface plasmon resonance on a BIACore instrument as described (Neri et al. (1997) Nature Biotechnol., 15, 1271-1275). Monomeric fractions of E1, A2 and G4 scFv fragments bound to ED-B with affinity in the  $10^7 - 10^8 M^{-1}$  range (~~Table 2~~) $107 - 108 M^{-1}$  range (~~Table 1~~).--.

Page 22, please replace the paragraph beginning at line 8, with the following:

--ScFv(E1) was selected to test the possibility of improving its affinity with a limited number of mutations of CDR residues located at the periphery of the antigen binding site (Figure 1A). We combinatorially mutated residues 31-33, 50, 52 and 54 of the antibody VH, and displayed the

corresponding repertoire on filamentous phage. These residues are found to frequently contact the antigen in the known 3D-structures of antibody-antigen complexes. The resulting repertoire of  $4 \times 10^8$  clones was selected for binding to the ED-B domain of fibronectin. After two rounds of panning, and screening of 96 individual clones, an antibody with 27-fold improved affinity was isolated (H10; Tables 1 and 2). Similarly to what others have observed with affinity-matured antibodies, the improved affinity was due to slower dissociation from the antigen, rather than by improved kon values (Schier et al. (1996). Gene, 169, 147-155, Ito (1995). J. Mol. Biol., 248, 729-732). The antibody light chain is often thought to contribute less to the antigen binding affinity as supported by the fact that both natural and artificial antibodies devoid of light chain can still bind to the antigen (Ward et al. (1989) Nature, 341, 544-546, Hamers-Casterman et al. (1993). Nature, 363, 446-448). For this reason we chose to randomize only two residues (33 and 50)~~(32 and 50)~~ of the VL domain, which are centrally located in the antigen binding site (Figure 1a) and often found in 3D structures to contact the antigen. The resulting library, containing 400 clones, was displayed on phage and selected for antigen binding. From analysis of the dissociation profiles using real-time interaction analysis with a BIACore instrument (Jonsson et al. (1991). BioTechniques, 11, 620-627) and koff measurements by competition experiments with electrochemiluminescent detection a clone (L19) was identified, that bound to the ED-B domain of fibronectin with a  $K_d=54$  pM Table 1~~(Tables 1 and 2)~~.

Page 23, please replace the paragraph beginning at line 1, with the following:

--Affinity maturation experiments were performed as follows. The gene of scFv(E1) was PCR amplified with primers LMB1bis (SEQ ID NOs 1-2, respectively) (5'-GCG GCC CAG CCG GCC ATG GCC GAG-3') and DP47CDR1for (5'-GA GCC TGG CGG ACC CAG CTC ATM NNM NNM NNGCTA AAG GTG AAT CCA GAG GCT G-3') to introduce random mutations at positions 31-33 in the CDR1 of the VH (for numbering: 28), and with primers DP47CDR1back (SEQ ID NOs 3-4, respectively) (5'-ATG AGC TGG GTC CGC CAG GCT CC-3') and DP47CDR2for (5'-GTC TGC GTA GTA TGT GGT ACC MNN ACT ACC MNN AAT MNN TGA GAC CCA CTC CAG CCC CTT-3') to randomly mutate positions 50,52,54 in

CDR2 of the VH. The remaining fragment of the scFv gene, covering the 3'-portion of the VH gene, the peptide linker and the VL gene, was amplified with primers DP47CDR2back (SEQ ID NOs 5-6, respectively) (5'-ACA TAC TAC GCA GAC TCC GTG AAG-3') and JforNot (5'-TCA TTC TCG ACT TGC GGC CGC TTT GAT TTC CAC CTT GGT CCC TTG GCC GAA CG-3') (94°C 1 min, 60°C 1 min, 72°C 1 min). The three resulting PCR products were gel purified and assembled by PCR (21) with primers LMB1bis and JforNot (94°C 1 min, 60°C 1 min, 72°C 1 min). The resulting single PCR product was purified from the PCR mix, double digested with NotI/NcoI and ligated into NotI/NcoI digested pDN332 vector. Approximately 9 µg of vector and 3 µg of insert were used in the ligation mix, which was purified by phenolisation and ethanol precipitation, resuspended in 50 µl of sterile water and electroporated in electrocompetent TGI E. coli cells. The resulting affinity maturation library contained  $4 \times 10^8$  clones. Antibody-phage particles, produced as described (Nissim et al. (1994). EMBO J., 13, 692-698) were used for a first round of selection on 7B89 coated imunotube (Carnemolla et al. (1996). Int. J. Cancer, 68, 397-405). The selected phages were used for a second round of panning performed with biotinylated ED-B, followed by capture with streptavidin coated magnetic beads (Dynal, Oslo, Norway; see previous paragraph). After selection, approximately 25% of the clones were positive in soluble ELISA (see previous chapter for experimental protocol). From the candidates positive in ELISA, we further identified the one (H10; ~~Table 1~~) with lowest koff by BIACore analysis (Jonsson et al. (1991), BioTechniques, 11, 620-627).--.

Page 23, please replace the paragraph beginning at line 31, with the following:

-- The gene of scFv(H10) was PCR amplified with primers LMB1bis and DPKCDR1for SEQ ID NO 7) (5'-G TTT CTG CTG GTA CCA GGC TAA MNN GCT GCT GCT AAC ACT CTG ACT G) to introduce a random mutation at position 3332 in CDR1 of the VL (for numbering: Chothia and Lesk (1987) J. Mol. Biol., 196, 901-917), and with primers DPKCDR1back (SEQ ID NOs 8-9, respectively) (5'-TTA GCC TGG TAC CAG CAG AAA CC-5') and DPKCDR2for (5'-GCC AGT GGC CCT GCT GGA TGC MNN ATA GAT GAG GAG CCT GGG AGC C-3') to introduce a random mutation at position 50 in CDR2 of the VL. The remaining portion of the

scFv gene was amplified with oligos DPKCDR2back (SEQ ID NO 10) (5'-GCA TCC AGC AGG GCC ACT GGC-3') and JforNot (94 C 1 min, 60 C 1 min, 72 C 1 min) The three resulting products were assembled, digested and cloned into pDN332 as described above for the mutagenesis of the heavy chain. The resulting library was incubated with biotinylated ED-B in 3% BSA for 30 min., followed by capture on a streptavidin-coated microtitre plate (Boehringer Mannheim GmbH, Germany) for 10 minutes. The phages were eluted with a 20 mM DTT solution (1,4-Dithio-DL-threitol, Fluka) and used to infect exponentially growing TG1 cells.--.

Page 24, please replace the paragraph beginning at line 19, with the following:

--The above mentioned anti-ED-B antibody fragments were then produced inoculating a single fresh colony in 1 liter of 2 x TY medium as previously described in Pini et al. ((1997), J. Immunol. Meth., 206, 171-182) and affinity purified onto a CNBr-activated sepharose column (Pharmacia, Uppsala, Sweden), which had been coupled with 10 mg of ED-B containing 7B89 recombinant protein (Carnemolla et al. (1996). Int. J. Cancer, 68, 397-405). After loading, the column was washed with 50 ml of equilibration buffer (PBS, 1 mM EDTA, 0.5 M NaCl). Antibody fragments were then eluted with triethylamine 100 mM, immediately neutralised with 1 M Hepes, pH 7, and dialysed against PBS. Affinity measurements by BIACore were performed with purified antibodies as described (Neri et al. (1997). Nature Biotechnol., 15 1271-1275) [FIG. 4]. Band-shift analysis was performed as described (Neri et al. (1996). Nature Biotechnology, 14, 385-390), using recombinant ED-B fluorescently labelled at the N-terminal extremity (Carnemolla et al. (1996). Int. J. Cancer, 68, 397-405, Neri et al. (1997). Nature Biotechnol., 15 1271-1275) with the infrared fluorophore Cy5 (Amersham) [FIG. 4]. BIACore analysis does not always allow the accurate determination of kinetic parameters for slow dissociation reactions due to possible rebinding effects, baseline instability and long measurement times needed to ascertain that the dissociation phase follows a single exponential profile. We therefore performed measurements of the kinetic dissociation constant koff by competition experiments (Neri et al. (1996), Trends in Biotechnol., 14, 465-470) [FIG. 4]. In brief, anti-ED-B antibodies (30 nM) were incubated with biotinylated ED-B (10 nM) for 10

minutes, in the presence of M2 anti-FLAG antibody (0.5 µg/ml)~~(0.5 µg/ml)~~ and polyclonal anti-mouse IgG (Sigma) which had previously been labelled with a rutenium complex as described (Deaver, D. R. (1995). Nature, 377, 758-760). To this solution, in parallel reactions, unbiotinylated ED-B (1 µM) was added at different times. Streptavidin-coated dynabeads, diluted in Origen Assay Buffer (Deaver, D. R. (1995). Nature, 377, 758-760) were then added (20 µl, 1 mg/ml), and the resulting mixtures analysed with a ORIGEN Analyzer (IGEN Inc. Gaithersburg, Md. USA). This instrument detects an electrochemiluminescent signal (ECL) which correlates with the amount of scFv fragment still bound to the biotinylated ED-B at the end of the competition reaction. Plot of the ECL signal versus competition time yields a profile, that can be fitted with a single exponential with characteristic constant koff [FIG. 4; Table 12].--.

Page 34, please delete in its entirety.

Pages 35 – 39, please renumber to pages 34 – 38.

Renumbered page 34, first line, please replace with the following:

--Table 12:--.